

## DESCRIPTION

### NOVEL HIGH-THROUGHPUT SCREENING METHOD of DRUG for BIOACTIVE PROTEIN

#### Technical Field

[0001]

The present invention relates to a safe and quick method for searching a drug to a bioactive protein, in particular, an inhibitor, using a cell-free protein synthesis system with the use of a wheat embryo extract. Specifically, a protein is synthesized in vitro safely, handy, and efficiently from a gene in a same reaction system, based on base sequence information in a pathogen including viruses, without being constrained by regulations for recombinant experiments from biohazard issues and others. Furthermore, the present invention relates to a method for screening a drug, in particular, an inhibitor, by using a cell-free protein synthesis system which can co-translationally trace the activity of a bioactive protein, wherein in a reaction system similar to an intracellular environment, the method uses the reactivities to the bioactive protein's autodigestion, recognition of a substrate, function, structure and others in a translation or folding process as an indicator.

#### Background Art

[0002]

As a method to conduct intracellular protein synthesis *ex vivo*, such as in a test tube, an *in vitro* cell-free protein synthesis method has been widely researched, wherein ribosome, for example, and other components necessary for protein synthesis are extracted from an organism (herein sometimes referred to as "wheat embryo extract for cell-free protein synthesis") and used (Patent Documents 1, 2, 3, 4 and 5).

[0003]

The cell-free protein synthesis system is a useful method, which keeps performance comparable to the system in a living cell concerning accuracy and speed of translation reaction, and can provide a target protein without carrying out a complicated purification step. Therefore, the more effective application of the synthesis system for industrial uses needs, in addition to increasing synthesis efficiencies, providing a wheat embryo extract-containing solution for use in synthesis and a ready-made type wheat embryo extract-containing solution steadily and at a retained high quality.

[0004]

Meanwhile, the infection caused by a pathogen including viruses, even though it has been overcome to some extent by progress in chemotherapy and so on, is still a big threat to human, as can be seen from year-by-year

epidemic of influenza, the occurrence of novel infectious diseases such as AIDS, E. Coli O157, SARS, West Nile virus, and Ebola hemorrhagic fever or from the reoccurrence of tuberculosis which was believed to have been overcome for a while. Under these circumstances, it has recently been demonstrated that a protease inhibitor to HIV virus is effective as an anti-AIDS drug, and it is put into practical use. The research for an infectious disease treatment medicament to target a protease which is indispensable for viral proliferation is believed to be more important in future.

[0005]

At present, in a commonly conducted research on a protease inhibitor, an E. Coli or the like is treated by genetic recombination technology to produce a protease protein, which is then coexisted with the substrate protein and a test substance to search for an effective substance as an inhibitor using a substrate cleaving activity as an indicator. However, the production of a protein from a pathogen by genetic recombination needs a large scale experiment facility at a level of P3 and P4 is required and subjects itself to many regulatory constraints. In addition, a researcher can not perfectly be exempted from the risk to be infected with those pathogens even if protected by sufficient facilities.

[0006]

Further, according to a conventional method, a drug, which is determined by an in vitro experiment to have a protease inhibitory activity, can in some cases not inhibit viral proliferation. It is suggested that the drug according to the conventional method is purified to complete its folding so that it may have a different structure to affect substrate-specificity.

[0007]

In addition, recent infectious diseases of concern, SARS (severe acute respiratory syndrome) is an emerging infectious disease appeared in early 2003, and has affected 8,447 people and deprived 811 lives worldwide as of the end of June in 2003. SARS is characterized by high fever, malaise, ague, headache and dyspnoea, and progressed to cause interstitial pneumonia, requiring intubation and mechanical respiration. Currently, the fatality rate in the SARS infected is as high as approximately 15%, and it is believed that the infection route is mainly by direct contact although other routes cannot be fully excluded. From the many evidences, as a novel coronavirus exists in persons infected with SARS, the responsible pathogen for SARS is believed to be a novel coronavirus (SARS-CoV). Further, an essential proteinase for SARS-CoV's self-reproduction is found out, and it is pointed out that the catabolic enzyme shows important functions in the viral life cycle while causing SARS's symptoms (Nonpatent

Document 2). Therefore, a proteinase for SARS-CoV (SARS 3CL<sup>pro</sup>) is considered to be a drug target effective in SARS treatment, and diverse researches have been conducted.

[0008]

Until now, the following pharmacological researches on SARS have been proceeded by a number of groups. Study on candidate inhibitors of SARS 3CL<sup>pro</sup> in molecular modeling for the three dimensional structural analysis of SARS 3CL<sup>pro</sup>'s crystalline structure, which is a primary proteinase for SARS-CoV (Nonpatent Document 1). Study on candidate drugs to SARS in molecular modeling for the binding mechanism between SARS 3CL<sup>pro</sup> and ligands (Nonpatent Document 2). They have not experimentally examined SARS 3CL<sup>pro</sup> and inhibitors thereto, and not reached to practical application.

[0009]

Further, as the study of an inhibitor to the pathogen using a cell-free protein synthesis system, a method for screening an inhibitor to inhibit autodigestion is proposed wherein a rhinovirus protease is expressed in a cell-free protein synthesis system derived from rabbit's reticulocytes. However, that screening method is not practical because, for example, to screen enormous candidate drugs using a cell-free protein synthesis system derived from rabbit's reticulocytes needs a plenty of synthesis solution to obtain, has a problem in cost, and

further requires a tracer experiment to execute using a radioisotope for the screening detection due to the slight amount of expressed protein in the synthesis system of interest (Nonpatent Document 3).

[0010]

In spite of the above situation in SARS study, currently no effective drug for treating SARS has been available. The reasons are as follows: SARS-CoV has been recently identified to be the pathogen of SARS. Furthermore, to study such a highly-fatal virus as SARS pathogen, the experiment is difficult to manage because of constraints from biohazard. Moreover, it has been believed that a bioactive protein expressed from a decoded genomic sequence is difficult to screen while it keeps activity, because of problems from its conformation and posttranslational modification.

[0011]

[Patent Document 1] Japanese Patent Application  
Laid-open No. Hei 6-98790

[Patent Document 2] Japanese Patent Application Laid-open  
Hei 6-225783

[Patent Document 3] Japanese Patent Application Laid-open  
Hei 7-194

[Patent Document 4] Japanese Patent Application Laid-open  
Hei 9-291

[Patent Document 5] Japanese Patent Application Laid-open

Hei 7-147992

[Patent Document 6] International Patent Application  
PCT/US98/25742

[Nonpatent Document 1] Anand K, Ziebuhr J, Wadhwani P,  
Mesters JR, Hilgenfeld R.

Coronavirus main proteinase (3Clpro) structure:basis for  
design of anti-SARS drugs.Science. 2003 Jun  
13;300(5626):1763-7. Epub 2003 May 13.

[Nonpatent Document 2] Kuo-Chen Chou, Dong-Qing Wei, and  
Wei-Zhu Zhong.

Binding mechanism of coronavirus main proteinase with  
ligands and its implication to drug design against  
SARS.Biochemical and Biophysical Research Communications  
308 (2003) 148-151

[Nonpatent Document 3] BEVERLY A. HEINZ, JOSEPH TANG, JEAN  
M. LABUS, FREDERICK W. CHADWELL, STEPHEN W. KARLDOR, AND  
MARLYS HAMMOND

Simple In Vitro Translation Assay To Analyze Inhibitors of  
Rhinovirus Proteases

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan.1996, p.267-270  
Disclosure of the Invention

[0012]

An object of the present invention is to provide a  
safe and quick means for screening a drug to a bioactive  
protein, in particular, an inhibitor, using a cell-free  
protein synthesis system with the use of a wheat embryo

extract. Another object of the present invention is to provide a non-conventional means for screening a drug to a bioactive protein, in particular, an inhibitor, using a cell-free protein synthesis system with the use of a wheat embryo extract which allows co-translational trace of the activity of the bioactive protein, wherein in a reaction system similar to an intracellular environment, the means uses the reactivities of the bioactive protein such as autodigestion, substrate recognition, function, structure and others in a translation or folding process as an indicator.

[0013]

The present inventors have strenuously studied to solve the above problems and finally completed the present invention by constructing a synthesis system of a bioactive protein with its bioactivities kept in a cell-free protein synthesis means with the use of a wheat embryo, and constructing a system for screening an inhibitor candidate to SARS 3CL<sup>Pro</sup> as an example using the synthesis system.

[0014]

Therefore, the present invention is composed of:

1. A method for searching a drug to a bioactive protein using a cell-free protein synthesis means with the use of a wheat embryo extract solution comprising at least steps 3) to 5) of the following steps:
  - 1) synthesizing a gene comprising a gene encoding the

bioactive protein, wherein the step is based on base sequence information of the bioactive protein gene,

2) synthesizing an mRNA from the gene synthesized in step 1),

3) synthesizing the bioactive protein using a cell-free protein synthesis system with the use of a wheat embryo extract solution, using the mRNA synthesized in step 2) as a translation template or the gene synthesized in step 1) as a transcription template,

4) determining the reactivity of a candidate drug to the bioactive protein by adding the candidate drug to the cell-free protein synthesis system with the use of a wheat embryo extract solution, and

5) screening a drug to the bioactive protein by using the reactivity as an indicator.

2. The method for searching a drug according to the preceding 1, wherein the indicator of reactivity to the bioactive protein is based on the reactivity to bioactive protein's autodigestion.

3. The method for searching a drug according to the preceding 1, wherein the indicator of reactivity to the bioactive protein is based on the reactivity to the bioactive protein's substrate recognition.

4. The method for searching a drug according to the preceding 1, wherein the indicator of reactivity to the bioactive protein is based on the bioactive protein's

autodigestion in a folding process, inhibition or termination of folding, or induction of misfolding.

5. The method for searching a drug according to the preceding 1, wherein the reactivity of the candidate drug to the bioactive protein is selected from any one, two or more of the following:

1) a reaction of inhibiting or terminating a synthesis of the bioactive protein's mRNA in a transcription process,

2) a reaction of inhibiting and/or antagonizing autodigestion at one, two or more autodigestion site(s) in the bioactive protein,

3) a reaction of inhibiting and/or antagonizing the recognition of a substrate at one, two or more site(s) for recognizing a substrate in the bioactive protein,

4) a reaction of inhibiting or terminating the synthesis of the bioactive protein in a translation process,

5) a reaction of inhibiting or terminating the autodigestion or folding of the bioactive protein in a folding process, or a reaction of inducing misfolding,

6. The method for searching a drug according to any one of the preceding 1 to 5, wherein the steps 3) to 5) or 2) to 5) of the preceding 1 are conducted in a single reaction system.

7. The method for searching a drug according to any one of the preceding 1 to 6, wherein the wheat embryo extract solution is a cell-free protein synthesis means with a wheat

embryo extract from which an endosperm and a low molecular synthesis inhibitor are substantially removed.

8. The method for searching a drug according to any one of the preceding 1 to 7, wherein the bioactive protein is a protein associated with pathogenic proliferation.

9. The method for searching a drug according to any one of the preceding 1 to 8, wherein the bioactive protein is a protease.

10. The method for searching a drug according to any one of the preceding 1 to 9, wherein the bioactive protein gene is a gene derived from any one of the following:

1) a double-stranded DNA virus, 2) a singled-stranded DNA virus, 3) a positive-stranded RNA virus, 4) a negative-stranded RNA virus, 5) a double-stranded RNA virus, 6) a retrovirus, and 7) a hepadnavirus.

11. The method for searching a drug according to any one of the preceding 1 to 10, wherein the bioactive protein is any one of the following:

1) an RNA polymerase, 2) a DNA polymerase, 3) a helicase, 4) a coat protein, and 5) a capsid protein.

12. The method for searching a drug according to any one of the preceding 1 to 11, wherein the bioactive protein gene is derived from SARS.

13. A drug provided by the method for searching a drug according to any one of the preceding 1 to 12.

14. A reagent kit used in the method for searching a drug

according to any one of the preceding 1 to 12.

15. An oligonucleotide primer to amplify a SARS 3CL<sup>pro</sup> protein-encoding DNA.

16. The oligonucleotide primer according to the preceding 15, comprising any one of nucleotides represented by SEQ.ID.Nos: 6-21.

17. A SARS 3CL<sup>pro</sup> protein-encoding DNA synthesized by using the oligonucleotide primer according to the preceding 15 or 16.

18. The SARS 3CL<sup>pro</sup> protein-encoding DNA according to the preceding 17, represented by SEQ.ID.No:1.

19. A SARS 3CL<sup>pro</sup> protein synthesized using a cell-free system with the use of a wheat embryo extract solution, using the DNA according to the preceding 17 or 18.

20. The SARS 3CL<sup>pro</sup> protein according to the preceding 19, sustaining a protease activity.

[0015]

The present invention has provided a safe and quick means for screening a drug to a bioactive protein, in particular, an inhibitor, using a cell-free protein synthesis system with the use of a wheat embryo extract. Furthermore, the present invention has succeeded in providing a means for screening a drug to a bioactive substance protein, in particular, an inhibitor, using the reactivities to bioactive protein's autodigestion, recognition of a substrate, or function, structure or

others in a translation and/or folding process as an indicator.

Description of the Preferred Embodiment

[0016]

(1) Preparation of Wheat Embryo Extract-containing Solution for Cell-Free Protein Synthesis

A cell-free protein synthesis system used in the present invention uses a wheat embryo extract. Herein, the cell-free protein synthesis system means an in vitro synthesis method carried out by extracting components including, for example, ribosome, which is an intracellularly-equipped protein translation apparatus, from wheat embryo, and adding the transcription template or translation template, nucleic acids as the substrate, amino acids, energy source, various ions, buffer, and other useful factors to this extract solution. The system includes a system using RNA as template (hereinafter it is sometimes referred to as "cell-free translation system") and a system using DNA and reacting by the further addition of an enzyme necessary for transcription such as RNA polymerase (hereinafter it is sometimes referred to as "cell-free transcription/translation system"). The cell-free protein synthesis system in the present invention includes both of above described cell-free translation system and cell-free transcription/translated system.

[0017]

A wheat embryo extract-containing solution used in the present invention is commercially available as PROTEIOS™ (from TOYOB0). As a preparation method of a wheat embryo extract solution, for the isolation method of wheat embryo, the method described in, for example, Johnston, F. B. et al., *Nature*, 179, 160-161 (1957) may be used, and for an extraction method from the isolated wheat embryo for a wheat embryo extract-containing solution, for example, the method described in Erickson, A. H. et al., (1996) *Meth. In Enzymol.*, 96, 38-50 may be used. Besides them, the methods described in Japanese Patent Applications No.2002-23139 and No.2002-231340 may be mentioned.

[0018]

A wheat embryo extract suitable for use in the present invention will be purified by removing almost all endosperm comprising components inhibiting protein synthesis function (components capable to influence the mRNA, tRNA, protein translation factors, ribosome and others, for example, tritin, thionine, ribonuclease and inhibit the function), which a material cell itself does contain or retain. Herein, the purified product from which almost all endosperm is removed means a wheat embryo extract remained after the removal of a endosperm portion to the extent that ribosome will not be substantially deadenylated, and further the extent that ribosome will not be substantially deadenylated means an extent that the ribosome has a

deadenylation rate of less than 7% and preferably 1% or less.

[0019]

The wheat embryo extract described above may comprise a protein derived from a wheat embryo extract-containing solution and a protein separately added according to need. In view of the preservation stability in a freeze-dried state, its usability and the like, the composition before freeze-dried has preferably a protein content of, but not limited in particular to, 1 to 10 weight % of the total composition, and more preferably 2.5 to 5 weight %, and further the lyophilized composition after freeze-dried has preferably a protein content of 10 to 90 weight % of the total lyophilized composition, and more preferably 25 to 70 weight %. Now, the protein content herein can be calculated by measuring its absorbance (260, 280 and 320 nm).

[0020]

(2) Reduction of Deliquescent Substances in Solution Comprising Wheat Embryo Extract

The wheat embryo extract-containing solution described above contains deliquescent substances such as potassium acetate and magnesium acetate from an extraction solvent or a buffer for use in gelfiltration after extraction. Thus, the wheat embryo extract-containing solution is used to prepare a translation reaction solution,

which is then directly lyophilized to make a dry formulation with a trouble such as deliquescence, resulting in deterioration in quality of the formulation. The deterioration in quality means that the formulation is not completely dissolved in water, thereby to reduce synthesis activities in protein synthesis reaction. Therefore, the deliquescent substances in the wheat embryo extract-containing solution may be decreased in concentration to give no influence on the quality of the lyophilized formulation. The specific methods for decreasing deliquescent substances include, for example, a gel filtration method wherein a gel support is equilibrated beforehand with a deliquescent substance-reduced or free solution to use, or a dialysis method. By using these methods, the deliquescent substances in a solution finally prepared for translation reaction should be reduced to have a final concentration of 60 mM or less. Specifically, potassium acetate in the solution finally prepared for translation reaction should be reduced to have a concentration of 60 mM or less, and preferably 50 mM or less. Furthermore, the lyophilized formulation has preferably a deliquescent substance content of 0.01 parts by weight or less to 1 part by weight of the protein contained in the formulation, and particularly preferably 0.005 parts by weight or less to keep the preservation stability in the freeze-dried state.

[0021]

(3) Elimination of contaminant microbes

As the wheat embryo extract-containing solution can be contaminated with spores of microbes, in particular a filamentous bacterium (mold) and the like, these microbes are preferred to be eliminated. Microbial proliferation may be seen in particular in a long-term (a day or higher) cell-free protein synthesis reaction, and hence is important to inhibit. The means of eliminating microbes preferably includes, but is not limited to, a filter for filter sterilization to use. The filter may not be limited in pore size to a particular one in so far as it can eliminate contamination-suspected microbes, but has usually a pore size of 0.1 to 1  $\mu\text{m}$ , and preferably 0.2 to 0.5  $\mu\text{m}$ .

[0022]

(4) Method to Remove Low Molecular Synthesis Inhibitors from Wheat Embryo Extract-containing Solution

In addition to the foregoing operations, a step for removing low molecular synthesis inhibitors can be added anywhere in the preparation of the wheat embryo extract-containing solution to make the solution suitable for cell-free protein synthesis of a bioactive protein with more preferable effects. A wheat embryo extract-containing solution prepared by substantially removing an endosperm component contains low molecular synthesis inhibitors having a protein synthesis inhibitory

activity (this may be referred to as "low molecular synthesis inhibitor"). Thus, the removal of them may provide a wheat embryo extract-containing solution having a high protein synthesis activity. Specifically, the removal is conducted by fractionally removing low molecular synthesis inhibitors from the components of a wheat embryo extract-containing solution through the differences in their molecular weights. The low molecular synthesis inhibitor can be fractionally removed to have a smaller molecular weight than the least factor among those factors necessary for protein synthesis that are contained in the wheat embryo extract-containing solution. Specifically, the inhibitor may be fractionally removed to have a molecular weight of 50,000 to 14,000 or less, or preferably of less than 14,000. As the method for removing low molecular synthesis inhibitors from a wheat embryo extract-containing solution, a method already known per se, for example, dialysis using a dialysis membrane, gelfiltration, or ultrafiltration can be used. Among them, dialysis is preferred in view of, for example, the easiness of supplying an internal solution with materials.

[0023]

As a dialysis membrane for use in the removing operation of low molecular synthesis inhibitors through dialysis, the one which can remove a molecule having a molecular weight of 50,000 to 12,000 may be mentioned,

specifically, a recyclable cellulose membrane (from Viskase Sales, Chicago) which can remove a molecule having a molecular weight of 12,000 to 14,000, Spectra/Pore 6 (from SPECTRUM LABOTRATORIES INC., CA, USA) which can remove a molecule having the molecular weight of 50,000, and the like may preferably be used. A suitable amount of the wheat embryo extract-containing solution will be put toward the one-side of such dialysis membrane, and then dialysis be conducted by a conventional method. The dialysis is preferred to be conducted for 30 minutes to 24 hours.

[0024]

While removing low molecular synthesis inhibitors, if an insoluble substance is produced in a wheat embryo extract-containing solution, inhibiting this production (hereinafter, this may be referred to as "the stabilization of a wheat embryo extract-containing solution") allows the wheat embryo extract-containing solution finally prepared or a solution for translation reaction to have a higher protein synthesis activity. As a specific method of stabilizing a wheat embryo extract-containing solution or a solution for translation reaction, there is mentioned a method wherein low molecular synthesis inhibitors described above are removed from a wheat embryo extract-containing solution or a solution for translation reaction with at least a high energy phosphate compound such as ATP or GTP (hereinafter, they may be referred to as

"stabilization component") contained. As the high energy phosphate compound, ATP may preferably be used. Further, the removal may be preferably carried out from the solution with ATP and GTP, and more preferably ATP, GTP, and 20 kinds of amino acids contained.

[0025]

The solution may be supplied with these stabilization components, incubated, and then subjected to the process for removing low molecular synthesis inhibitors. Alternatively, the stabilization component may be added also to an external solution for dialysis, and then the solution is subjected to dialysis to remove low molecular synthesis inhibitor. Advantageously, the stabilization component in the external solution for dialysis, even if decomposed during dialysis, can be constantly supplemented with the fresh stabilization component. This approach can be applied to gelfiltration and ultrafiltration used to give the same effect. The supports they use are equilibrated with a filtration buffer with a stabilization component contained, supplied with a wheat embryo extract-containing solution or a solution for translation reaction with a stabilization component contained, and the supplied with the above-described buffer to filtrate.

[0026]

The amount of a stabilization component to add and the time of stabilization to treat may be selected as

appropriate depending on the kind of a wheat embryo extract-containing solution and the method of preparation. As the selection method, there may be mentioned a method wherein a wheat embryo extract-containing solution is supplied with various stabilization components in amount and kind on a trial basis, and subjected to the step for removing low molecular synthesis inhibitors after an appropriate hour to give a treated wheat embryo extract-containing solution, which is then centrifuged to separate into the soluble compartment and the insoluble component, thereby to select a case resulting in a less amount of the insoluble component. Alternatively, a method is preferred wherein the treated wheat embryo extract-containing solution is used to carry out cell-free protein synthesis, thereby to select a case resulting in a high protein synthesis activity. Further, a method is mentioned wherein stabilization component is added in an external solution for dialysis, and then wheat embryo extract-containing solution is subjected to dialysis for an appropriate time, thereby to select based of the amount of the insoluble component in the solution thus obtained or the protein synthesis activity of the solution thus obtained.

[0027]

As one example of the stabilizing condition of a wheat embryo extract-containing solution thus selected,

specifically, if dialysis is carried out for the step of removing low molecular synthesis inhibitors, there is mentioned a method wherein the wheat embryo extract-containing solution and the external solution for dialysis are supplied with 100  $\mu$ M to 0.5 mM of ATP, 25  $\mu$ M to 1 mM of GTP and 25  $\mu$ M to 5 mM of each 20 kinds of amino acids and then subjected to dialysis for 30 minutes to an hour or more. The temperature for dialysis may be any temperature so far as it does not deteriorate the protein synthesis activity of the wheat embryo extract-containing solution and allows dialysis. Specifically, the lowest temperature is a temperature at which the solution will not freeze, usually -10°C, and preferably -5°C. The highest temperature is a limit temperature which gives no bad influence on the solution used in dialysis, 40°C, and preferably 38°C.

[0028]

In addition, if low molecular synthesis inhibitors are removed after a wheat embryo extract-containing solution is prepared, the solution needs no further the above stabilization component to add.

[0029]

(5) Method for Decreasing Concentration of Reducing Agent in Wheat Embryo Extract-containing Solution

The wheat embryo extract-containing solution, which contains a reducing agent at a decreased concentration, is

used to execute cell-free protein synthesis, allowing production of a target bioactive protein which has an intramolecularly formed disulfide bond. For a method for decreasing a reducing agent in a wheat embryo extract-containing solution, there is used a method wherein a step of decreasing a reducing agent is employed anywhere in the steps for the preparation of the wheat embryo extract-containing solution. The reducing agent should be decreased to have so a concentration in the finally prepared wheat embryo extract-containing solution that the solution may be used to execute translation reaction, allowing synthesis of a bioactive protein which has an intramolecularly formed disulfide bond to sustain. Dithiothreitol (hereinafter, this may be referred to as "DTT") as a reducing agent is decreased to have a final concentration of 20 to 70  $\mu$ M, and preferably 30 to 50  $\mu$ M in the final solution for translation reaction prepared from a wheat embryo extract-containing solution. 2-mercaptoethanol is decreased to have a final concentration of 0.1 to 0.2 mM in the final solution for translation reaction. Glutathione/oxidized glutathione is decreased to have a final concentration of 30 to 50  $\mu$ M/1 to 5  $\mu$ M in the final solution for translation reaction. The specific concentration of a reducing agent is not limited to those described above and varies appropriately depending on the protein to synthesize or the kind of a cell-free

protein synthesis system to use.

[0030]

The method of selecting the optimal concentration range of a reducing agent is not limited in particular, and, for example, there is mentioned a selection method based on the effect of a catalyst for disulfide bond exchange reaction. Specifically, solutions for translation reaction are prepared from a wheat embryo extract-containing solution at various concentrations of a reducing agent, and then supplied with a enzyme capable of catalyzing disulfide bond exchange reaction to synthesize a bioactive protein having an intramolecular disulfide bond. In addition, as a control experiment, the same protein synthesis is carried out using the same solutions for translation reaction supplied with no enzyme capable of catalyzing disulfide bond exchange reaction. Then, the soluble component of a bioactive protein to synthesize is separated by a method such as centrifugation. The reaction solution, wherein this soluble component has a share of 50% (solubilization 50%) or more in the total and further has been increased by the addition of an enzyme capable of catalyzing disulfide bond exchange reaction, can be determined to be a suitable reaction solution for synthesizing a bioactive protein with an intramolecular disulfide bond retained. Furthermore, within the concentration range of a reducing agent selected based on

the effect of the catalyst for disulfide bond exchange reaction as above described, the concentration of the reducing agent which can synthesize the highest amount of the bioactive protein can be selected as more preferable concentration range.

[0031]

For specific methods for decreasing a reducing agent, there is used a method wherein a wheat embryo extract-containing solution is prepared to be free from a reducing agent, and then supplied with a reducing agent to have an above described concentration range together with necessary components for a cell-free protein synthesis system, or a method wherein a reducing agent is removed from a solution for translation reaction derived from a wheat embryo extract-containing solution to be within the concentration range described above. As a wheat embryo extract-containing solution for cell-free protein synthesis requires a high degree of reduction condition to extract, a method wherein a reducing agent is removed from the solution after extraction is easier to execute. As a method for removing reducing agent from a wheat embryo extract-containing solution, there is mentioned a method using a gelfiltration support. Specifically, for example, there is mentioned a method wherein Sephadex G-25 column is beforehand equilibrated with an appropriate buffer containing no reducing agent, and then fed with a wheat

embryo extract-containing solution to pass through.

[0032]

(6) Preparation of Solution for Translation Reaction

The wheat embryo extract-containing solution prepared as described above is supplied with a nuclease inhibitor, various ions, a substrate, an energy source and the like necessary for protein synthesis (hereinafter, they may be referred to as "additives for a solution for translation reaction") and an mRNA encoding a target bioactive protein, which acts as a translation template, and, if desired, a stabilizer which comprising a component selected from the group consisting of inositol, trehalose, mannitol, and sucrose-epichlorohydrin copolymer to prepare a solution for translation reaction. The concentrations of components to add may be provided from a compounding ratio well known per se.

[0033]

The additives for a solution for translation reaction, specifically, include amino acids acting as substrate, an energy source, various ions, a buffer, an ATP-regenerating system, a nuclease inhibitor, a tRNA, a reducing agent, polyethylene glycol, a 3', 5'-cAMP, a folate, an antimicrobial, and the like. Further, concerning each concentration, preferably, ATP is contained at 100  $\mu$ M to 0.5 mM, GTP at 25  $\mu$ M to 1 mM and 20 kinds of amino acids at their respective 25  $\mu$ M to 5 mM. They can be selected

and combined for use as appropriate according to the translation reaction system. Specifically, a wheat embryo extract, which is used for a wheat embryo extract-containing solution, is supplied with 20 mM of HEPES-KOH (pH 7.6), 100 mM of potassium acetate, 2.65 mM of magnesium acetate, 0.380 mM of spermidine (from Nacalai Tesque), respectively 0.3 mM of 20 kinds of L-amino acids, 4 mM of dithiothreitol, 1.2 mM of ATP (from Wako Pure Chemical Industries, Ltd.), 0.25 mM of GTP (from Wako Pure Chemical Industries, Ltd.), 16 mM of phosphocreatine (from Wako Pure Chemical Industries, Ltd.), 1000U/ml of RNase inhibitor (from TAKARA) and 400µg/ml of creatine kinase (from Roche), to dissolve sufficiently, followed by adding the mRNA translation template supporting an mRNA encoding a target bioactive protein.

[0034]

Herein, the mRNA encoding the target bioactive protein has a structure wherein the sequence encoding a bioactive protein capable of being synthesized in a cell-free protein synthesis system consisting of wheat embryo is linked in the downstream of both an appropriate sequence recognized by RNA polymerase and further a sequence having a function to activate translation. The sequence recognized by RNA polymerase includes T3 or T7 RNA polymerase promoter. Further, in a cell-free protein synthesis system, as a sequence enhancing a translation

activity, there may be preferably used a sequence having a structure wherein  $\Omega$  sequence, Sp6 or the other is linked to the 5'-upstream of the coding sequence.

[0035]

(7) Bioactive Protein

The bioactive protein according to the present invention is a protein synthesized based on the base sequence of an organism's gene and having specific functions derived from an organism. Further, the base sequence for a bioactive protein is not necessarily identical to the base sequence of an organism, but may be a base sequence into which modifications such as deletion, substitution, addition, insertion are appropriately introduced as long as the protein has specific functions. For example, a protein synthesized on the basis of the base sequence of the gene of a pathogen including viruses and having specific functions derived from the pathogen may be mentioned. As kinds of pathogens including viruses, double-stranded DNA viruses, singled-stranded DNA viruses, positive-stranded RNA viruses, negative-stranded RNA viruses, double-stranded RNA viruses, retroviruses, hepadnaviruses and the like may be mentioned but not limited in particular. Further, the bioactive protein of a specific function includes proteins associated with pathogenic proliferation, in more detail, a protease, a helicase, an RNA polymerase and the like, and also a coat

protein and a capsid protein which are involved in the structural formation of viruses, but is not limited in particular.

[0036]

In the present invention, the transcription/translation of a bioactive protein in a cell-free synthesis system with the use of a wheat embryo extract allows easier synthesis of a bioactive protein which sustains the tertiary structure in a almost native state and its activities, and further the simultaneous addition of a candidate drug in the synthesis system with the reaction allows easier screening for a useful candidate drug which targets autodigestion, recognition of a substrate, and the reaction in translating or folding process.

In the Examples, bioactive proteins were synthesized based on the amino acid sequence (GenBank's accession number AY274119) of the major proteinase of SARS-CoV which has previously disclosed, SARS 3CL<sup>Pro</sup>. Therefore, on the basis of amino acid sequence the codons of each amino acid were examined and a primer was designed by selecting GC-rich codons so that the annealing site of the primer became shorter. The whole gene was synthesized using this primer through Inverse PCR. The SARS 3CL<sup>Pro</sup> was synthesized by obtaining mRNA from this gene and translating the mRNA as template in cell-free synthesis with the use of a wheat

embryo extract. Then, based on the fact that the resulting SARS 3CL<sup>pro</sup> sustained a protease activity, a drug screening system employing SARS 3CL<sup>pro</sup> was constructed. However, this is disclosed just by way of a preferable illustration and not by way of limitation. The amino acids sequence of SARS 3CL<sup>pro</sup> shown in Examples was presented in SEQ. ID. NO: 32.

[0037]

(8) Method of Synthesizing Protein Using Cell Extract Solution for Cell-Free Protein Synthesis

The wheat embryo extract solution, that is, a cell extract solution prepared as above for cell-free protein synthesis can be dissolved in a dissolving solution which is supplied with a deliquescent substance and water to have a concentration appropriate for protein synthesis reaction, and put into a selected system or apparatus already known per se to take place protein synthesis. As a system or apparatus for protein synthesis, there are mentioned a method such as the batch method (Pratt, J. M. et al., *Transcription and Translation*, Hames, 179-209; B. D. & Higgins, S. J., eds, IRL Press, Oxford (1984)) wherein a translation reaction solution in which the cell extract solution for cell-free protein synthesis of the present invention is dissolved is kept at an appropriate temperature for the synthesis, a cell-free protein synthesis system in a continuous manner (Spirin, A. S. et

al., *Science*, 242, 1162-1164 (1988)) wherein amino acids, an energy source and others necessary for a cell-free protein synthesis system are fed into the reaction system continually, a dialysis method (Kigawa et al., The 21st The Molecular Biology Society of Japan, WID6), and a method wherein a solution containing amino acids, an energy source and others necessary for a cell-free protein synthesis system is overlaid onto a solution for translation reaction (bilayer system: Sawasaki, T., et al., 514, 102-105 (2002)).

[0038]

Herein, when the cell extract for cell-free protein synthesis is used at a decreased concentration of a reducing agent, a solution for supplying amino acids, an energy source and others necessary for a cell-free protein synthesis system also is adjusted to have the same concentration of the reducing agent. Furthermore, the translation reaction is conducted in the presence of an enzyme capable of catalyzing disulfide bond exchange reaction to allow high efficient synthesis of a bioactive protein which retains an intramolecular disulfide bond. As the enzyme capable of catalyzing disulfide bond exchange reaction, for example, a protein disulphide isomerase may be mentioned. The amount of these enzymes to add to a cell-free translation system described above may be selected as appropriate depending on the kind of enzyme.

Specifically, the solution for translation reaction, that is, a wheat embryo extract-containing solution which is extracted from wheat embryo and contains DTT as a reducing agent at 20 to 70  $\mu$ M and preferably 30 to 50  $\mu$ M is supplied with a protein disulfide isomerase to have a final concentration of 0.01 and 10  $\mu$ M, and preferably 0.5  $\mu$ M in the reaction solution for translation. Further, the addition is preferable in timing before the initiation of translation reaction in view of the efficiency of the formation of a disulfide bond.

[0039]

(9) Screening Method

A screening method in a cell-free system with the use of a wheat embryo extract solution according to the present invention is a non-conventional method for screening a candidate drug comprising at least steps 3) to 5) of steps 1) to 5) as shown below. The conventional method for screening a candidate drug has been a method which mainly aims at, for example, the activities or structure of a matured protein. A cell-free protein synthesis system with the use of a wheat embryo extract solution of the present invention permits screening a drug to a bioactive protein in a state such as <1>, and <3> through <5> described below. Furthermore, combination of different indicators allows screening drugs which are different in mechanism of pharmacological effects and drugs having integral effects,

as well as drugs which are appropriate for cocktail therapy against pathogens.

[0040]

A method for searching a drug to a bioactive protein using a cell-free protein synthesis means with the use of a wheat embryo extract solution according to the present invention comprises the following steps 1) to 5):

1) Synthesizing a gene comprising a gene encoding the bioactive protein, wherein the step is based on base sequence information of the bioactive protein gene.

A labeling base sequence such as GFP, GUS and GST, and further the recognition sequence for the bioactive protein may also be added to the bioactive protein gene to provide the base sequence of the bioactive protein gene.

2) Synthesizing an mRNA from the gene synthesized in step 1).

The transcription process can be a previously known art.

3) Synthesizing the bioactive protein using the cell-free protein synthesis system with the use of a wheat embryo extract solution, using the mRNA synthesized in step 2) as a translation template or the gene synthesized in step 1) as a transcription template.

The cell extract-containing solution for cell-free protein synthesis is added by, for example, Pipetman and/or the channel pipette of an automatic pipetting device respectively to different wells of a container divided into

a plurality of regions in an amount appropriate to the well. Subsequently, a solution comprising substances necessary for protein synthesis, the translation template or the transcription template, and a stabilizer are added to each well by, for example, Pipetman and/or the channel pipette of an automatic pipetting device in an amount of requirement to synthesize the bioactive protein.

4) Determining the reactivity of a candidate drug to the bioactive protein or bioactive protein's mRNA by adding the candidate drug to the cell-free protein synthesis system.

The candidate drug may be added at any time point in the bioactive protein synthesis system, during which a transcription step, a translation step, an autodigestion process, a substrate recognition step, and a folding step should occur. A drug can be added simultaneously with the translation step , to allow screening by targeting the folding step of the bioactive protein or the change in folding, which is a non-conventional screening means using a novel system.

5) Screening a drug to the bioactive protein by using the reactivity of a candidate drug to the bioactive protein as an indicator.

The method for determining the reactivity of a candidate drug to the bioactive protein qualitatively or quantitatively includes any method which may be served as long as it can detect the reactivity, and is not limited

in particular. Specifically, a bioactive protein's site for recognizing a substrate or a bioactive protein to synthesize in a cell-free protein synthesis system may be labeled with a label to trace the reactivity to the bioactive protein qualitatively and quantitatively by using the label as a marker. As the labeling means in this instance, common means using such as deuterium, radioisotope, fluorescent substances, and color source substances may be mentioned. Further, a candidate drug, which can use the reactivity to the translation template for the bioactive protein as an indicator, can be judged qualitatively and quantitatively for the reactivity to the mRNA for the bioactive protein from the amount of expression of the bioactive protein synthesized in the cell-free protein synthesis system or the presence or absence of the expression of the labeled specific protein.

Further, a cell-free protein synthesis manner with the use of a wheat embryo extract liquid according to the present invention allows synthesis of a bioactive protein with bioactivity sustained. Therefore, the synthesized bioactive protein allows screening a candidate drug without the protein subjected to a purification step, and thus steps 3) through 5) or 2) through 5) indicated above can be conducted in a single reaction system.

[0041]

Now below, the detection (determination) method of

the present invention for using reactivity of a candidate drug to a bioactive protein as an indicator will be explained, and they are offered by way of illustration and not by way of limitation.

<1> Translation Reaction of mRNA for Bioactive Protein

In the translation process of the mRNA for a bioactive protein, the amount of the bioactive protein synthesized is detected by the addition of the candidate drug. The insertion of a marker sequence into the base sequence for a bioactive protein may provide the detection by the amount of the marker detected. Further, using SDS-PAGE, the presence or absence of the band of the bioactive protein, which is synthesized depending on the presence or absence of the candidate drug will be detected.

<2> Matured Bioactive Protein

Protease: A labeled substance comprising a protease cleavage recognition site is contacted with protease and a candidate drug, and then the labeled product is detected, or subjected to SDS-PAGE to detect the presence or absence and the position of the band for a peptide generated by protease cleavage.

RNA polymerase: a radiolabeled or fluorescence-labeled ribonucleotide and a candidate drug are reacted using DNA or RNA as template to detect no fresh RNA synthesized, wherein the product is separated by PAGE or capillary, and then detected by autoradiogram if a radiolabel is used, or

excited by a laser or a mercury lamp to detect by wavelength through a polarized light filter if fluorescence used.

DNA polymerase: a DNA or RNA primer, radiolabeled or fluorescence-labeled deoxyribonucleotide, and a candidate drug are reacted using the DNA or the RNA as a template to detect no fresh DNA synthesized, wherein the product is separated by PAGE or capillary, and then detected by autoradiogram if a radiolabel is used, or excited by a laser or a mercury lamp to detect by wavelength through a polarized light filter if fluorescence used.

Helicase: a single-strand specific nuclease and a candidate drug are reacted using a radiolabel or fluorescence-labeled double-stranded DNA or RNA as a template to detect whether the template becomes shorter or not, wherein the product is separated by PAGE or capillary, and then detected by autoradiogram if a radiolabel is used, or excited by a laser or a mercury lamp to detect by wavelength through a polarized light filter if fluorescence used.

### <3> Autodigestion Reaction of Bioactive Protein

A labeled substance comprising the autocleavage recognition site of a bioactive protein is contacted with the bioactive protein and a candidate drug, and then the labeled product is detected, or subjected to SDS-PAGE to detect the presence or absence and the position of the band for a peptide generated by protease cleavage. Alternately,

a protein is synthesized to comprise an autocleavage recognition site sequence in the linker between the labeled substance and the bioactive protein, and the labeled product is detected.

<4> Bioactive Protein's Substrate Recognition Reaction

A substance comprising a substrate recognition sequence with a labeling substance contained, a bioactive protein and a candidate drug are contacted to detect change in fluorescence intensity, or subjected to SDS-PAGE to detect the contrast (including the presence or absence of the band) and the position of the band of the bioactive protein which has recognized the substrate.

<5> Folding Reaction of Bioactive Protein

It is detected from change in the tertiary structure using, for example, NMR and CD that a labeled bioactive protein and a candidate drug are reacted to terminate folding or to induce misfolding. Alternatively, it can be detected by reactivity with a monoclonal or a single chain antibody.

[0042]

In order to screen an inhibitor candidate to SARS protease activity, a cell-free protein synthesis means with the use of a wheat embryo extract solution of the present invention is used to synthesize the SARS protease ( $SA3CL^{pro}$ ) in a state for sustaining protease activity. Then, a substance (GFP-RS-GUS) containing cleavage site sequence RS with a labeling substance GFP contained,  $SA3CL^{pro}$  and an

inhibitor candidate are contacted to detect change in GFP fluorescence intensity, or subjected to SDS-PAGE to detect the presence or absence and the position of the band of GFP-RS-GU or GFP. Thereby, an inhibitor can be searched by using the decrease in GFP fluorescence intensity because of the inhibition of the RS cleavage in GFP-RS-GUS, or the change in contrast or the change in position of a GFP-RS-GU or GFP band (including the presence or absence of the band) as an indicator.

[0043]

For a candidate drug according to the present invention, various kinds of compound libraries well known per se can be selected. The cell-free protein synthesis system with the use of a wheat embryo extract solution, which is not a screening system of living cell system using E. Coli and the like, can be used not only for a candidate drug affecting cell proliferation and a candidate drug hardly incorporated into a cell, but also for a candidate drug poorly soluble in water because the system can synthesize a protein in the presence of an organic solvent. Specifically, low molecular compound libraries, which are commercially available, may be mentioned.

[0044]

Further, a reagent kit used in a method for searching a drug to a bioactive protein using a cell-free protein synthesis means with the use of a wheat embryo extract

solution according to the present invention comprises at least a cell extract solution for cell-free protein synthesis. Furthermore, the reagent is used to synthesize a bioactive protein in a container divided into a plurality of regions, allowing high-throughput screening a candidate drug.

[0045]

The present invention will be explained in details below with following examples, and the scope of the present invention will not be limited by these examples.

EXAMPLE 1

[0046]

Detection of SARS 3CL Protease Activity

(1) Cloning of Genetic DNA of SARS 3CL Protease

A synthesized DNA, which had been designed from the information of amino acid sequence of a SARS 3CL protease gene, was used as a primer and pBSIIKS+ was used as a template to conduct Inverse PCR, thereby to fabricate SARS 3CL protease gene. Namely, the sense primer S1 (SEQ.ID.No:6) and the antisense primer A1 (SEQ.ID.No:14) were used to position in the both sides of HindIII site of pBSIIKS+, to conduct Inverse PCR, followed by treating with exonuclease I to remove unreacted primers. The obtained PCR product as template, S1 primer, S2 primer (SEQ.ID.No:7) which overlapped each other with A1 primer's end by respective 15mers, and A2 primer (SEQ.ID.No:15) were used to conduct

Inverse PCR. The obtained PCR product as template, S2 primer, S3 primer (SEQ.ID.No:8) which overlapped each other with A2 primer by respective by 15mers, and A3 primer (SEQ.ID.No:16) were used to conduct Inverse PCR, thereby to amplify DNA. Subsequently, S3, A3, S4 (SEQ.ID.No:9), A4 (SEQ.ID.No:17), S5 (SEQ.ID.No:10), A5 (SEQ.ID.No:18), S6 (SEQ.ID.No:11), A6 (SEQ.ID.No:19), S7 (SEQ.ID.No:12), A7 (SEQ.ID.No:20), S8 (SEQ.ID.No:13) and A8 (SEQ.ID.No:21), which were designed to overlap by 15 mers in the same manner, were used to repeat Inverse PCR, thereby to amplify DNA. This plasmid was extracted with phenol/chloroform, cleaved with a restriction enzyme, HindIII (from NEB) and purified with GENE CLEAN II Kit (from Funakoshi Co., Ltd.). This purified restriction enzyme fragment was circularized by self-ligation. This circularized plasmid was transformed, plated onto the LB medium containing ampicillin (100ppm) and cultivated overnight at 37°C. The colony of DNA having the aimed fragment length was selected from the colonies obtained by PCR, and finally the sequence was confirmed by sequencing. The colony having the aimed DNA fragments was downsized and this plasmid was amplified in E. Coli. Thereafter, to obtain the plasmid alone, it was purified using GenElute™ Plasmid Miniprep Kit (from SIGMA). The obtained vector (pBS-SA3CL<sup>Pro</sup>) was cleaved with restriction enzymes, Xhol and BamH1 (from NEB), and pEU-E01-MCS vector was also cleaved with Xhol and BamH1, purified with GENE

CLEAN II Kit (from Funakoshi Co., Ltd), and then ligated in the same manner to obtain the plasmid {pEU-E01- SA3CL<sup>pro</sup> (SEQ.ID.No:1)}. This plasmid was subjected to PCR using SPu primer (SEQ.ID.No:22) and AODA2303 primer (SEQ.ID.No:23) to obtain a transcription/translation template. Furthermore, a mutant, wherein the 145th cysteine which was believed to share the center of SARS 3CL protease activity was altered to alanine, pEU-E01-SA3CL<sup>pro</sup> (C145A) (SEQ.ID.No:3) was constructed by the same method as indicated above.

[0047]

(2) Cloning of Genetic DNA of Substrate GFP-RS-GUS

While pEU-E01-GUS vector was subjected to Inverse PCR using an antisense primer (SEQ.ID.No:24) comprising E01 and Xhol sequences and a sense primer (SEQ.ID.No:25) comprising Pst1 sequence and the start codon of GUS, PEU3-GFP vector was subjected to PCR using a sense primer (SEQ.ID.No:26) comprising Xhol sequence and the start codon of GFP, and a primer (SEQ.ID.No:27) comprising the cleavage site sequence (RS) of SA3CL<sup>pro</sup> and a portion of GFP sequence. The respective PCR products were extracted with phenol/chloroform and cleaved using Xhol and Pst1 (from NEB). The cleaved DNA was purified with GENE CLEAN II Kit (from Funakoshi Co., Ltd), the plasmid was circularized by ligation and transformed, the aimed colony was selected in the same manner as in (1) and downsized, and the plasmid

{pEU-GFP-RS-GUS (SEQ.ID.No:2)} was amplified in E. Coli. Thereafter, to obtain the plasmid alone, it was purified using GenElute™ Plasmid Miniprep Kit (from SIGMA). The obtained pEU-E01-GUS vector was subjected to PCR using SPu primer and AODA2303 primer.

[0048]

(3) Cloning of Fusion Gene from GFP-RS-SA3CL<sup>pro</sup> and GFP-RS-SA3CL<sup>pro</sup> (C145A)

PCR was carried out using pBS-SA3L<sup>pro</sup> plasmid as a template, RS-SA3L<sup>pro</sup>-S1 (SEQ.ID.No:28), which was a sense primer comprising Pst1 site and a portion of RS sequence, and M13 primer (SEQ.ID.No:29). This PCR product was extracted with phenol/chloroform and cleaved with restriction enzymes, Pst1 (from NEB) and BamH 1 (from NEB). Further, GFP-RS-GUS plasmid was cleaved with Pst1 (from NEB) and BamH 1 (from NEB) in the same manner. These two kinds of cleaved DNAs were purified with GENE CLEAN 2 Kit (from Funakoshi Co., Ltd), the plasmid was circularized by ligation and transformed, the aimed colony was selected in the same manner as in (1) and downsized, and this plasmid was amplified in E. Coli. Thereafter, to obtain plasmid {pEU-GFP-RS-SA3CL<sup>pro</sup> (SEQ.ID.No:5)} alone, it was purified using GenElute™ Plasmid MinipreKit (from SIGMA). The obtained GFP-RS-SA3CL<sup>pro</sup> vector was subjected to PCR using SPu primer, AODA2303 primer. Furthermore, GFP-RS-SA3CL<sup>pro</sup> (C145A) was constructed by inserting mutant SA3CL<sup>pro</sup> (C145A),

wherein the 145th cysteine which was also believed to share the center of SARS 3CL protease activity was altered to alanine.

[0049]

(4) Cloning of GST-RS-SA3CL<sup>pro</sup> Fusion Gene

PCR was conducted using pEU-E01-GSTN2 plasmid as a template, GST-RS-SA3L<sup>pro</sup>sen (SEQ.ID.No:30), which was a sense primer comprising Xho1 site and the start codon of GST, and GST-RS-SA3L<sup>pro</sup> anti (SEQ.ID.No:31) which was an antisense primer comprising Pst 1 site and a portion of GST sequence. This PCR product was extracted with phenol/chloroform and cleaved with restriction enzyme, Xho 1 (from NEB) and Pst 1 (from NEB). Further, GFP-RS-GUS plasmid was cleaved with Pst1 (from NEB) and BamH 1 (from NEB) in the same manner. These two kinds of DNAs was purified with GENE CLEAN 2 Kit (from Funakoshi Co., Ltd), the plasmid was circularized by ligation and transformed, the aimed colony was selected in the same manner as in (1) and downsized, and this plasmid {pEU-GST-RS-SA3CL<sup>pro</sup> (SEQ.ID.No:4)} was amplified in E. Coli. Thereafter, to obtain the plasmid alone, it was purified using GenElute<sup>TM</sup> Plasmid MinipreKit (from SIGMA). The obtained GST-RS-SA3CL<sup>pro</sup> vector was subjected to PCR using Spu primer, AODA2303 primer.

[0050]

(5) Preparation of Wheat Embryo Extract Solution

The seeds of Chiho wheat produced in Hokkaido and/or those of Chikugoizumi wheat produced in Ehime were fed into the mill (from Fritsch: Rotor Speed Mill pulverisette Type 14) at a rate of 100 g/min. and pulverized gently at a speed of 8,000 rpm. After collecting a fraction containing wheat embryo having germinability with a sieve (sieve opening from 0.7 to 1.00 mm), selection by flotation with the mixture of carbon tetrachloride and cyclohexane (volume ratio: carbon tetrachloride: cyclohexane = 2.4: 1) was conducted to recover a floating fraction containing wheat embryo having germinability, then organic solvents were evaporated to remove at room temperature, and then mixed impurities such as seed coats were removed by blowing at room temperature to obtain a crude wheat embryo fraction. Wheat embryo was determined in this crude wheat embryo fraction by visual inspection and sorted out with a bamboo rod. The obtained wheat embryo fraction was suspended in distilled water at 4°C, and washed with a wash solution in the ultrasonic cleaner until white color became clear. Then, it was suspended in 0.5 v% solution of Nonidet (from Nacalai Techtonics) P40 and washed with a wash solution in the ultrasonic cleaner until white color became clear to obtain wheat embryo. The preparation of a wheat embryo extract-containing solution was conducted in accordance with a conventional method (Erickson, A. H. et al., (1996) Meth. In Enzymol., 96, 38-50). The following operations

were conducted at 4°C. First, wheat embryo frozen with liquid nitrogen was finely ground in a mortar. 1ml of an extraction solvent prepared by the partial modification of Pattersons' method per 1g of the obtained powder, 80 mM of HEPES-KOH (pH 7.6), 200 mM of potassium acetate, 2 mM of magnesium acetate, 4 mM of calcium chloride, and 0.6 mM of each 20 kinds of L-amino acids, and 8 mM of threitol (respectively) were added to obtain their respective final concentrations, and then stirred carefully without developing foam. A supernatant obtained by centrifugation at 30,000 x g for 15 min. was recovered as wheat embryo extract solution and subjected to gel filtration with Sephadex G-25 column (from Amerham Pharmacia Biotech), which was previously equilibrated with a solution whose final concentrations were (respectively) as follows: 40 mM of HEPES-KOH (p) 7.6, 100 mM of potassium acetate, 5 mM of magnesium acetate, 0.3 mM of each 20 kinds of L-amino acids, and 4 mM of threitol. The concentration of a wheat embryo extract-containing solution thus obtained was adjusted to have an optical density (O.D.) (A260) 170 to 250 (A260/A280=1.5) at 260 nm.

[0051]

#### (6) Fabrication of Translation Template

The PCR products cloned in (1), (2), (3) and (4) were used respectively as a transcription template for in vitro transcription. For transcription, 25  $\mu$ l of reaction

system was prepared to have a final concentration of as follows respectively: 80 mM of Hepes-KOH, 16 mM of magnesium acetate, 2 mM of spermidine (from Nacalai Techtonics), 10 mM of DTT, 3 mM of NTP (from Wako Pure Chemical Industries, Ltd.), 1 U/μl of SP6 RNA polymerase, 1 U/μl of Rnase Inhibitor (from TAKARA) and the 10% PCR product. These reaction solutions were incubated for three hours at 37°C, and then the mRNAs {SA3CL<sup>pro</sup>, SA3CL<sup>pro</sup> (C145A), GFP-RS-GUS, GFP-RS-SA3CL<sup>pro</sup>, GST-RS-SA3CL<sup>pro</sup>} were purified by ethanol precipitation.

[0052]

(7) Protein Synthesis Using Cell-free Protein Synthesis System Using Wheat Embryo Extract Solution (bilayer system)

Proteins (GFP-RS-GUS, GFP-RS-SA3CL<sup>pro</sup> and GST-RS-SA3CL<sup>pro</sup>) were synthesized using a bilayer system. First, 125 μl of the external solution for dialysis (the respective final concentrations were as follows: 30 mM of HEPES-KOH (pH 7.8), 100 mM of potassium acetate, 2.7 mM of magnesium acetate, 0.4 mM of spermidine (from Nacalai Techtonics), 0.25 mM of each 20 kinds of L-amino acids, 2.5 mM of threitol, 1.2 mM of ATP, 0.25 mM of GTP, 16 mM of phosphocreatine (from Wako Pure Chemical Industries, Ltd.)) was placed into a microtiter plate, the wheat embryo extract-containing solution prepared in (5) indicated above was added to provide a reaction solution for protein synthesis to have a final optical density (O.D.) (A260) of

60 (the final concentrations were respectively as follows: 30 mM of HEPES-KOH (pH 7.8), 100 mM of potassium acetate, 2.7 mM of magnesium acetate, 0.4 mM of spermidine (from Nacalai Techtonics), 0.25 mM of each 20 kinds of L-amino acids, 2.5 mM of threitol, 1.2 mM of ATP, 0.25 mM of GTP, 16 mM of phosphocreatine (from Wako Pure Chemical Industries, Ltd.) and 400 µg/ml of creatine kinase (from Roche)), and in 25 µl of this solution, the mRNAs (GFP-RS-GUS, GFP-RS-SA3CL<sup>Pro</sup> and GST-RS-SA3CL<sup>Pro</sup>) prepared in (6) indicated above were suspended, overlaid without disrupting the boundary surface of the reaction solution for protein synthesis, and incubated for 18 hours at 26°C to conduct protein synthesis.

[0053]

(8) Protein Synthesis Using Cell-free Protein Synthesis System Using Wheat Embryo Extract Solution (dialysis)

Proteins {SA3CL<sup>Pro</sup> and SA3CL<sup>Pro</sup> (C145A)} were synthesized through dialysis. 50 µl of the wheat embryo extract-containing solution used in (5) was added into dialysis cup MWCO 12000 (from Bio Tech), 700 µl of the external solution for dialysis was placed into Maruemu™ container (the final concentrations of both the wheat embryo extract-containing solution and external solution for dialysis, were same as those in (7)), and then incubated for a day at 26°C with supplying proteins' substrates, amino acids, ATP and the like for energy source to conduct protein

synthesis.

[0054]

(9) RS Sequence Cleavage by SARS 3CL Protease and Activity Detection

SA3CL<sup>pro</sup> and SA3CL<sup>pro</sup> (C145A) synthesized by dialysis (8) and the protein of GFP-RS-GUS, which was labeled with <sup>14</sup>C-Leu by bilayer system (7), (the final concentration of <sup>14</sup>C-Leu was 20  $\mu$ Ci/ $\mu$ l) were mixed in their equal amounts and incubated for two hours at 37°C (Fig. 1A). Thereafter, to this mixture, Sample Buffer (the final concentrations were respectively as follows: 50 mM of Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate, 1%  $\beta$ -mercaptoethanol, 10% glycerol and 0.2% BPB (from Nacalai Techtonics)) was added, and the resultant was heated at 98°C for 5 minutes followed by cooling rapidly with ice-cold water. This sample was subjected to SDS-PAGE using 12.5% SDS gel at 25 mA for 80 minutes. The RI-labeled protein was detected using an imaging plate and BAS-2500 (from Fuji Photo Film Co., Ltd.) (Fig. 1B). Further, after the protein synthesis of SA3CL<sup>pro</sup> and GFP-RS-GUS by dialysis, they were mixed in their equal amounts to perform Native PAGE, and the fluorescence was detected by Dark Reader (from BM Equipment Co., Ltd.) (Fig. 2)

[0055]

Referring to Fig. 1B, SA3CL<sup>pro</sup> was cleaved within a cleavage site {RS (PPQTSITSAVLQ $\downarrow$ SGFRKMAFPSGKV) } of the

linker between GFP and GUS, but mutant SA3CL<sup>pro</sup> (C145A) was not cleaved. Further, it was found that fluorescent substance GFP had a nature that its fluorescence intensity would become remarkably intensified by cleavage. Referring to Fig. 2, it was shown that the increase of concentration of SA3CL<sup>pro</sup> intensified GFP fluorescence intensity which showed the amount of GFP-RS-GUS cleaved. From this, it was found that as SA3CL<sup>pro</sup> activity was proportional to GFP fluorescence intensity, the inhibitory effect of a candidate drug capable of inhibiting the activity of a bioactive protein could be measured by fluorescence intensities.

[0056]

(10) Detection of Autolysis Activity by SA3CL<sup>pro</sup> from GST-RS-SA3CL<sup>pro</sup> and GFP-RS-SA3CL<sup>pro</sup>

To 10  $\mu$ l of synthesis solutions of GFP-RS-SA3CL<sup>pro</sup> and GST-RS-SA3CL<sup>pro</sup> (the final concentration of  $^{14}$ C-Leu was 12  $\mu$ Ci/ $\mu$ l) both of which were labeled with  $^{14}$ C-Leu by bilayer system (7), 5  $\mu$ l of 3 x Sample Buffer was mixed (Fig. 3A, Fig. 4A) to detect RI-labeled protein as described in (9) (Fig. 3B, Fig. 4B). As a method for detecting GST-RS-3CL<sup>pro</sup>, on the initiation of synthesis, the sample of GST-RS-3CL<sup>pro</sup> was collected every minutes, separated on SDS-PAGE, and then autoradiographically examined. Further, as a method for detecting GFP-RS-SA3CL<sup>pro</sup>, the sample was collected after 20 hours of reaction, separated on SDS-PAGE, and

autoradiographically examined (Fig. 4B). GFP-RS-SA3CL<sup>Pro</sup> and GFP-RS-SA3CL<sup>Pro</sup> (C145A) were synthesized by bilayer system, subjected to Native PAGE, and the fluorescence was detected by Dark Reader (from BM Equipment Co., Ltd.) (Fig. 5).

[0057]

Referring to Fig. 3B, autocleavage was found to occur in the cleavage site (RS) which had been designed to position in the linker part between SA3CL<sup>Pro</sup> itself and GST fused to its N-terminal end. Furthermore, within 30 minutes after the initiation of synthesis of GST-RS-SA3CL<sup>Pro</sup>, it was found that SA3CL<sup>Pro</sup> cleaved the RS positioned between GST and itself. From this, it was found that even in the folding process wherein SA3CL<sup>Pro</sup> had not become a fully matured protein (finished folding), the protein's autodigestion had already occurred. Referring to Fig. 4B, it was found that SA3CL<sup>Pro</sup>'s autocleavage occurred not only in cleavage site (RS) designed to position in the linker part between SA3CL<sup>Pro</sup> itself and GFP fused to its N-terminal end, but the products cleaved in sites other than the original cleavage site (RS) were detected (an arrow in the figure). Referring to Fig. 5, while SA3CL<sup>Pro</sup> was cleaved in cleavage site (RS) in the linker between GFP and itself, mutant SA3CL<sup>Pro</sup> (C145A) was not cleaved. From this combined with the results of Fig. 1B, it was found that in a cell-free system with the use of a wheat embryo extract solution

according to the present invention, bioactive proteins such as SA3CL<sup>Pro</sup> could be synthesized in a native state with sustaining its activity. Further, it was found that as SA3CL<sup>Pro</sup> was cleaved in the same manner as in Fig. 2, GFP's fluorescence intensity was intensified, and further SA3CL<sup>Pro</sup>'s activity was proportional to GFP fluorescence intensity, thereby the inhibitory effect of a candidate drug capable of inhibiting the activity of a bioactive protein could be measured by fluorescence intensities.

[0058]

From the results described above, it was found that the sensibility of protease activity of SA3CL<sup>Pro</sup> was different between during the folding process and after the termination of folding. Namely, protein synthesis in a cell-free synthesis system, after the initiation of synthesis, comprises also a protein on the way to folding, which sustains its activity as protease in spite of its incomplete folding, but it is suggested that the cleavage may occur at a position other than the original cleavage sites. In other words, it is believed that within an actual cell (in vivo), there may exist a bioactive protein which is on an incomplete folding of state but has a different cleavage site from the normal to sustain protease activity.

EXAMPLE 2

[0059]

Screening for Inhibitor Candidate to SARS Protease

(1) Synthesis by Bilayer System Using SARS Protease and Substrate Protein

Protein synthesis was conducted in accordance with a method described in EXAMPLE 1 (7) using SARS protease (SA3CL<sup>pro</sup>), a mutant thereof (SA3CL<sup>pro</sup> (C145A)) and the translation template of substrate GFP-RS-GUS described in EXAMPLE 1 (6). Herein, the final optical density (OD260) of a wheat embryo extract-containing solution was set to 120 and the final concentration of creatine kinase to 40 ng/μl. Further, synthesis reaction was conducted for 20 hours at 26°C with the upper layer set to 206 μl in amount of reaction solution and the lower layer to 20 μl.

[0060]

(2) Assay of Inhibitor Candidate

Each inhibitor candidate A and B was dissolved in 10% DMSO. Herein, inhibitor candidate A was a commercially available compound library from ASINEX Ltd., the accession No.AST6748415. Inhibitor Candidate B was a low molecular compound. 1 μl of SA3CL<sup>pro</sup> synthesis reaction solution described in the preceding paragraph (1), 3 μl of translation buffer (see EXAMPLE 1), 1 μl of 10% DMSO solution of each candidate A and B (Lane 3, Lane 4) (for control, 10%DMSO solution alone: Lane 2) were mixed. In addition, for negative control, in place of SA3CL<sup>pro</sup>, a mixture of 1 μl of SA3CL<sup>pro</sup> (C145A) synthesis reaction solution, 3 μl of translation reaction solution and 1 μl

of 10% DMSO solution (Lane 5) was used. After preincubating them for 10 minutes at 37°C, 5  $\mu$ l of GFP-RS-GUS synthesis reaction solution described in the preceding paragraph (1) was added and reacted for an hour at 37°C. Each reaction solution was analyzed by 12.5% Native PAGE and subjected to detection of the fluorescence intensity of GFP using Molecular Imager FXPro (from BioRad).

[0061]

The assay results of the inhibitor candidates were presented in Figure 6. In control (Lane 2), along with GFP-RS-GUS's band, GFP's band which was produced by SA3CL<sup>pro</sup> protease activity was also detected. SA3CL<sup>pro</sup> (C145A), which was used in negative control (Lane 5) and the mutant of the major responsible for protease activity, produced significantly less GFP because of the decreased protease activity. In the case of inhibitor candidate A (Lane 3) added, the production levels of GUS and GFP were observed to be as much as that of positive control (Lane 2), thereby it is believed that this compound may not have an inhibitory activity to SA3CL<sup>pro</sup> protease activity. In the case of inhibitor candidate B (Lane 4) added, GFP's band became weaker than that of positive control (Lane 2), therefore the fact that this compound had an inhibitory activity to SA3CL<sup>pro</sup> protease activity was presented. Then, Lane 1 was a fraction containing GFP-RS-GUS alone.

[0062]

From the results described above, the method for searching a drug to a bioactive protein using a cell-free protein synthesis means with the use of a wheat embryo extract of the present invention allowed screening for an inhibitor candidate to SARS protease activity.

[0063]

In a conventional screening system, mainly matured proteins after finishing folding were aimed. However, the system for screening a candidate drug to a protease on the folding process according to the present invention is a new system which has not been aimed by conventional screenings, and therefore it may be considered novel and useful. Furthermore, the screening system according to the present invention allows a means for screening a candidate drug which has reactivity to the structure or the function of a protein in the translation process, a protein in the autodigestion reaction process, a protein in the substrate recognition reaction process, and a protein in the folding reaction process, besides conventional matured proteins.

[0064]

This application claims priority from Japan Patent Serial No.2003-316081, which is incorporated herein by reference.

#### Brief Description of the Drawings

[0065]

Fig. 1A is a schematic representation of SARS protease

(3CL<sup>Pro</sup>), which is cleaving GFP-RS-GUS (substrate);

Fig. 1B is a figure of SDS-PAGE showing SA3CL<sup>Pro</sup> activity;

Fig. 2 is a figure of Native PAGE showing the differences in fluorescence intensity between before and after the GFP-GUS cleavage;

Fig. 3A is a schematic representation of SARS protease (3CL<sup>Pro</sup>) autocleavaging the cleavage site designed, wherein the protease was positioned at a knot connecting to GST, which was fused to the N-terminal of itself;

Fig. 3B is a figure of autoradiogram showing a fragment cleaved with GST-3CL<sup>Pro</sup> synthesized;

Fig. 4A is a schematic representation of SARS protease (3CL<sup>Pro</sup>) cleaving GFP which was fused to the N-terminal of the protease itself;

Fig. 4B is a figure of autoradiogram showing a fragment cleaved with GFP-3CL<sup>Pro</sup> synthesized;

Fig. 5 is a figure showing changes in fluorescence intensity by the inhibition of autolysis; and

Fig. 6 is a figure showing changes in fluorescence intensity by an inhibitor candidate.